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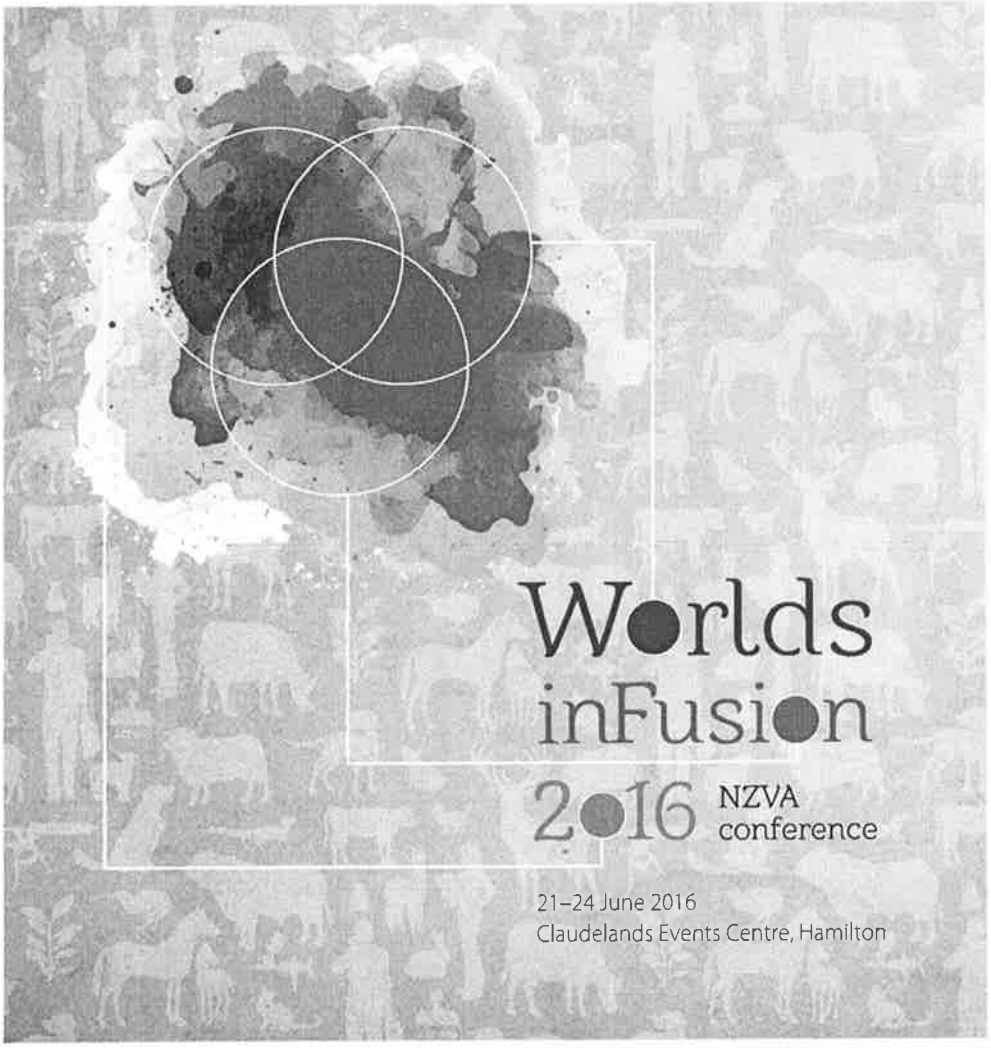

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Johne's disease diagnosis in New Zealand: An update

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Abstract

Johne's disease (JD) is a major production limiting disease of farmed ruminant species globally caused by infection with *Mycobacterium avium* subsp. *paratuberculosis* (*M. paratuberculosis* or *MAP*) and affects cattle, deer and sheep as well as farmed goats and alpaca throughout New Zealand. While this chronic wasting disease may be diagnosed immunologically by measuring antibody reactivity to bacterial infection, removal of all antibody positive animals may result in undue wastage as a significant proportion of test positive animals may have been infected but are not necessarily affected or diseased. Detection and stratification of diseased animals requires a test which is not host-specific but which is capable of measuring the amount of organisms shed in the faeces of infected animals and stratifying shedders accordingly; this is best achieved using a quantitative Polymerase Chain Reaction (qPCR) test. The importance of prompt identification and removal of MAP shedding livestock is recognised and endorsed by DairyNZ, JDRC and Johne's Management Ltd as the single most significant and immediate step towards reducing JD impact on affected farms and limiting spread of further infection. Unlike tuberculosis, caused by the closely related *Mycobacterium bovis*, eradication of JD at any level (on farm, regionally or nationally) is not considered feasible, achievable or likely and control efforts are instead focused upon cost-effectively managing clinical disease out of the herd.

Introduction

Johne's disease (JD) is a major production limiting disease of farmed ruminant species globally caused by infection with *Mycobacterium avium* subsp. *paratuberculosis* (*M. paratuberculosis* or MAP). In general, infection with MAP manifests as a chronic and progressive inflammatory intestinal disease, characterised by a slowly progressive wasting of the animal accompanied often, but not always, by decreased production and increasingly severe, watery diarrhoea. There is no treatment for JD and there is no cure. Johne's disease affects cattle, deer and sheep, as well as farmed goats and alpaca, throughout New Zealand at an estimated annual capital cost to the dairy, deer and sheep sector of NZ$40-88 million[1] and the urgent need for improved, rapid testing for JD, particularly for subclinical infection, continues to be recognised as being of high priority. Infection with MAP is considered widespread in New Zealand dairy herds, conservatively estimated to occur on more than 50% of farms although the true prevalence is likely to be even higher as many farmers may be unware of its presence within their livestock. In spite of high herd prevalence, MAP infection is manifest as a clinical disease causing significant production losses in a small proportion of infected herds and within-herd prevalence of clinically affected individuals is usually very low, although subclinical incidence may be significantly higher. Unlike Australia, Canada, Denmark, France, Ireland, The Netherlands, the United Kingdom and the United States, New Zealand has no organised surveillance programme and no coordinated control or management schemes in place, even though the New Zealand dairy industry is its biggest export earner with annual exports in excess of NZ$11 billion accounting for approximately 35% of the world trade in dairy products[2]. A current outline of best-practice guidelines for JD management has been prepared by the Johne's Disease Research Consortium (JDRC) alongside DairyNZ and is freely available[3]; a

[1]Johne's Disease Research Consortium; www.jdrc.co.nz/what.html
[2]New Zealand Trade and Enterprise; www.nzte.govt.nz/en/buy/our-sectors/food-and-beverage/dairy
[3]DairyNZ Toolbox for the management & control of Johne's disease in Dairy Cattle; www.dairynz.co.nz/animal/health-conditions/johnes-disease

supplementary guide to laboratory tests available in New Zealand and their appropriate application has also been prepared and will similarly be available through DairyNZ in the near future.

For all chronic, mycobacterial diseases in humans and animals, the development and validation of sensitive and specific diagnostic methods to diagnose infection and disease presents a significant challenge. Diagnostic tests are criticised as being insensitive and non-specific, a perceived deficit arising from the biology of mycobacterial infection and the chronology of the resultant immune responses triggered within the host, coupled with the existence of closely related and antigenically very similar mycobacterial species ubiquitous within the environment. These factors dictate that, however urgently they may be needed, the development of tests that more accurately and cost effectively diagnose MAP infection or JD in domestic animals is challenging. Since no existing single test satisfies all criteria in terms of sensitivity, specificity, turnaround, convenience of sample acquisition and cost-effectiveness, combinations of different tests are necessary to achieve optimal diagnosis. Commonly utilised *ante-mortem* diagnostic tests for JD include immunodiagnostic tests for serum antibody by ELISA or organism based tests to detect the presence of the bacterium, such as faecal culture or PCR. The specificity of immunodiagnostic tests may be compromised by common antigens shared by MAP, *M. bovis* and other saprophytic environmental mycobacteria which evoke an immune response in non-diseased animals. The sensitivity of serodiagnostic tests, particularly for subclinically infected animals in the early stages of JD, is also influenced by the dynamics of antibody production and the point at which a sample is assayed due to the predominantly cellular immune responses found in the early stages of disease, limiting the predictive value of the test (Gardner *et al.* 2011). While faecal culture on Herrold's egg yolk (HEY) medium has remained the definitive test for MAP infection this requires prolonged incubation periods of up to sixteen weeks and may be compromised by overgrowth by contaminating gut microflora (Bogli-Stuber *et al.* 2005, Clark *et al.* 2008, Harris and Barletta 2001, Irenge *et al.* 2009, Soumya *et al.* 2009). Radiometric liquid culture tests (e.g. BACTEC™) have been developed as a substitute for traditional faecal culture tests and have been widely adopted as they speed up the time to detect mycobacterial growth (Whittington 2010) although the withdrawal from the market in 2012 of the radiometric liquid medium most widely applied in a veterinary context BACTEC 12B (Becton Dickinson) has impacted severely upon the standing of this method (Whittington *et al.* 2013).

Internationally, rapid and quantitative measurement of MAP shedding in faeces of infected and affected animals by quantitative PCR (qPCR) is rapidly becoming the gold standard for JD diagnostic testing, supplanting older technologies based on culture, which is slow, expensive, low-throughput and, increasingly, obsolete (Slana and Kralik 2014). Because qPCR is comparatively labour intensive and expensive relative to ELISA it is not yet practical for whole herd screening and it has been used as a research tool rather than as a frontline diagnostic test in NZ. Recently faecal qPCR diagnostic assays have been applied in NZ in higher prevalence herds exhibiting high ELISA test prevalence where culling of all positives is not economically feasible. Used serially as a secondary test, qPCR is used to confirm the JD status of ELISA positive individuals and allows active shedders to be ranked in order of immediate risk, expediting prioritised culling of the worst affected cows according to available management resources while allowing alternative risk mitigation options to be applied for low shedders. In addition, the considerable dynamic range of faecal qPCR also lends itself to pooled sampling as high shedding individuals may be easily identified amongst low or non-shedders even at considerable dilution (Mita *et al.* 2016); this allows pooled sampling to be used for screening at reduced cost. Similarly, ELISA-negative cows may be re-screened by pooled qPCR for additional assurance in low prevalence herds. For whole-herd screening of

herds, particularly from milk samples, ELISA remains a preferred option due to cost and convenience.

In NZ, the Disease Research Laboratory (DRL, based in Dunedin) have for some years offered a custom ELISA-based test (Paralisa™) tailored for diagnosis of JD in farmed deer (Griffin *et al.* 2005, O'Brien *et al.* 2013) which was developed in response to poor performance in this species of commercial test kits designed for cattle. This serological test measures antibody titres in serum raised against MAP-specific antigens in the conventional manner using a detection antibody which is cross-reactive with both cervine and bovine IgG_1. In addition, DRL has developed a customised faecal qPCR test for quantitation of MAP shedding in dung samples, with results stratified into six categories: Not Detected, Background, Low, Moderate, High or Very High. The qPCR test for MAP quantitation has been validated for multiple ruminant species and may be applied to clinical samples from diverse host species. Here we describe a validation study of the performance of the Paralisa™ test applied to New Zealand dairy cattle in comparison to a second commercially available serum ELISA test kit marketed for use in cattle in NZ (IDEXX Paratuberculosis Screening Ab Test, IDEXX Laboratories, Inc., Westbrook, ME, USA), for a series of qPCR-determined shedding states and using qPCR as a gold-standard for the determination of sensitivity and specificity values.

Methods

Sample acquisition

Matched blood and faecal samples (n=1,069) from commercial dairy herds both with (n=23) and without (n=3) a history of Johne's disease were submitted for routine testing to the DRL laboratory, Dunedin, New Zealand.

ELISA

The Paralisa™ methodology was based on previously published parameters for ELISA immunoassays used to diagnose immune reactions in farmed deer to MAP infection (Griffin *et al.* 2005). This serological test quantifies antibody responses to two distinct protein antigens in parallel, a denatured antigen in the form of Purified Protein Derivative – Johnin (PPD-J) and a native protein in the form of Protoplasmic Antigen (PPA); final test results are arrived at by considering both test antigens in parallel. IDEXX ELISA assays (IDEXX Paratuberculosis Screening Ab Test, IDEXX Laboratories, Inc., Westbrook, ME, USA) were performed according to the instructions supplied by the kit manufacturer.

Faecal PCR

Quantitative measurement of faecal shedding was accomplished by quantitative faecal PCR as described by O'Brien *et al.* 2013 (O'Brien *et al.* 2013). Briefly, faecal samples submitted for laboratory testing (approximately 10g) were normalised by gravimetric dilution, homogenised to uniformity and filtered. One millilitre samples of filtrate were added to 2ml screwtop microcentrifuge tubes containing 400ul of a mixture of 0.1 and 0.5mm zirconia/silica beads (BioSpec, OK, USA) and centrifuged at 18kg for five minutes

to pellet suspended solids. The supernatant was removed and replaced by the supplied lysis buffer in a commercial faecal DNA extraction kit (Zymo Research Corp., CA, USA) and tubes mixed and placed in a boiling water bath for five minutes before being immediately bead beaten for five minutes at 1,750bpm in a GenoGrinder instrument (Spex SamplePrep, NJ, USA). Following bead beating the samples were again centrifuged at 18kg for five minutes to pellet undissolved particulates before 370ul of supernatant lysate was removed and processed for DNA extraction according to the manufacturer's protocol. Typical yields of recovered DNA were 5ug, approximately, of which 3ul (100ng approximately) was included in PCR reactions as template DNA. PCR amplification utilised the single-copy, species-specific MAP diagnostic target F57 (Tasara and Stephan 2005) using hydrolysis probe based real time PCR chemistry. Every assay included an internal amplification control labelled with a second reporter dye co-amplified alongside the diagnostic target in a duplex format to exclude false negative reactions and to disclose any inhibition of the reaction; biological and technical replicates and necessary positive and negative controls were also included. PCR amplification efficiency of both diagnostic target and co-amplified internal controls were typically >90%.

Quantitative judgements of MAP DNA titre in faecal samples was accomplished by using nucleic acid standard curves spanning seven serial log dilutions of MAP genomic DNA prepared from MAP strain 316f and results extrapolated and reported as 'MAP genome copies/ml' equivalents. DNA standards ranged from 16.5ng/ul to 1.65fg/ul; 3ul of standard DNA was utilised in each (20ul) PCR reaction such that, given a MAP genome size of 4.8Mbp (Li *et al.* 2005), these values equated to a topmost standard of 1×10^7 genomes/20ul (or 5×10^8 genomes/ml) down to a lowermost standard of 10 genomes/20ul reaction (or 5×10^2 genomes/ml). These standards spanned the range of MAP shedding observed in clinical samples and were linear in the assay over the seven logs (typically $R^2=0.999$). Assay results were broken down into six descriptive categories for reporting, ranging from Not Detected ($<10^2$ genomes/ml), Background ($\geq10^2$-$<10^3$ genomes/ml), Low ($\geq10^3$-$<10^4$ genomes/ml), Moderate ($\geq10^4$-$<10^5$ genomes/ml), High ($\geq10^5$-$<10^6$ genomes/ml) and Very High ($\geq10^6$ genomes/ml).

Using the qPCR method described here, this laboratory has participated in and passed proficiency panels of bovine faecal samples of known infection status and faecal culture titre, administered through the US National Veterinary Services Laboratory (NVSL), Ames, Iowa as part of an ongoing JD proficiency testing panel for diagnostic laboratories (Robbe-Austerman and Harris 2009). The NVSL JD Proficiency Panels are distributed annually to diagnostic and research laboratories in the US and internationally and are used to accredit testing services for JD diagnostic testing in the US. DRL are a USDA Animal and Plant Health Inspection Service accredited testing laboratory for Johne's disease (organism-based methods, direct PCR and pooled PCR)[4].

[4]APHIS-Approved Laboratories Johne's Disease Organism-Based Methods; www.aphis.usda.gov/animal_health/lab_info_services/downloads/ApprovedLabs_Johnes_organism.pdf

Results

Results for each of the ELISA methods and also their use in combination for various shedding states as determined by faecal PCR of matched faecal samples are reported in Table 1 and illustrated in Figure 1. The IDEXX serum ELISA test considered in isolation exhibited greater sensitivity than the Paralisa™ test for 4/5 of the shedding groups and comparable sensitivity for the lowest shedding group. At the lowest shedding category where $>10^2$ genomes/ml was considered positive the sensitivities for Paralisa and IDEXX were approximately equivalent at 0.57 and 0.56, respectively. Sensitivity at this threshold was raised by 18% to 0.74 on combination of the Paralisa and IDEXX data. Combination of IDEXX with the PPA component of the Paralisa alone raised the sensitivity of the assay by 14% (0.70), suggesting that the augmented diagnostic sensitivity observed was largely derived from the inclusion of the PPA antigen. At a shedding threshold of $>10^3$ genomes/ml the sensitivities for Paralisa and IDEXX were at 0.72 and 0.85, respectively. Sensitivity at this threshold was raised by a further 7% to 0.92 on combination of the Paralisa and IDEXX data; again the boosted sensitivity appeared to derive from the PPA contribution as a combination of IDEXX and PPA generated a similar sensitivity score of 0.92. At a shedding threshold of $>10^4$ genomes/ml there was no change to the sensitivity score recorded for the IDEXX test (0.85) whereas the Paralisa standalone score increased from 0.72 to 0.78. In combination the two tests had a sensitivity score of 0.91, an increase of 7% over the tests interpreted in isolation. At a shedding threshold of $>10^5$ genomes/ml the sensitivity of the IDEXX test was again unchanged at 0.85 while the Paralisa test dropped slightly to 0.73; the combination score was unchanged at 0.91 for this shedding category, an increase of 6% over standalone tests. In the final and highest shedding category of $>10^6$ genomes/ml, the calculated sensitivities for the IDEXX and Paralisa tests was 0.95 and 0.73, respectively, and in combination 0.95 with no further improvement over the $>10^5$ genomes/ml result.

		>102=POS; n=428	>103=POS; n=219	>104=POS; n=138	>105=POS; n=71	>106=POS; n=23
		≤102=NEG; n=641	≤103=NEG; n=850	≤104=NEG; n=931	≤105=NEG; n=998	≤106=NEG; n=1,046
IDEXX	Apparent prevalence	0.27(0.25,0.30)	0.27(0.25,0.30)	0.27(0.25,0.30)	0.27(0.25,0.30)	0.27(0.25,0.30)
	True prevalence	0.33(0.30,0.35)	0.17(0.14,0.19)	0.11(0.09,0.13)	0.06(0.05,0.07)	0.02(0.01,0.03)
	Sensitivity	0.56(0.50,0.61)	0.85(0.79,0.90)	0.85(0.77,0.91)	0.85(0.74,0.93)	0.95(0.76,1.00)
	Specificity	0.86(0.84,0.89)	0.84(0.81,0.86)	0.79(0.77,0.82)	0.76(0.73,0.79)	0.74(0.71,0.77)
	Pos. predictive value	0.66(0.60,0.72)	0.51(0.45,0.57)	0.33(0.28,0.39)	0.18(0.14,0.23)	0.07(0.04,0.10)
	Neg. predictive value	0.80(0.77,0.83)	0.97(0.95,0.98)	0.98(0.96,0.99)	0.99(0.98,0.99)	1.00(0.99,1.00)
	Pos. likelihood ratio	4.07(3.31,5.00)	5.33(4.53,6.28)	4.14(3.57,4.80)	3.59(3.09,4.18)	3.66(3.18,4.21)
	Neg. likelihood ratio	0.51(0.45,0.58)	0.18(0.12,0.25)	0.19(0.12,0.29)	0.19(0.10,0.35)	0.06(0.01,0.44)
ParalisaTM	Apparent prevalence	0.40(0.37,0.43)	0.40(0.37,0.43)	0.40(0.37,0.43)	0.40(0.37,0.43)	0.40(0.37,0.43)
	True prevalence	0.33(0.30,0.36)	0.17(0.15,0.19)	0.11(0.09,0.13)	0.06(0.05,0.07)	0.02(0.01,0.03)
	Sensitivity	0.57(0.52,0.62)	0.72(0.65,0.78)	0.78(0.69,0.85)	0.73(0.61,0.84)	0.73(0.50,0.89)
	Specificity	0.68(0.64,0.71)	0.66(0.63,0.69)	0.64(0.61,0.67)	0.62(0.59,0.65)	0.60(0.57,0.63)
	Pos. predictive value	0.46(0.42,0.51)	0.30(0.26,0.34)	0.21(0.17,0.25)	0.11(0.08,0.14)	0.04(0.02,0.06)
	Neg. predictive value	0.76(0.73,0.80)	0.92(0.90,0.94)	0.96(0.94,0.97)	0.97(0.96,0.98)	0.99(0.98,1.00)
	Pos. likelihood ratio	1.77(1.54,2.03)	2.11(1.86,2.40)	2.17(1.91,2.47)	1.92(1.63,2.27)	1.83(1.40,2.39)
	Neg. likelihood ratio	0.64(0.56,0.72)	0.43(0.34,0.54)	0.35(0.25,0.49)	0.43(0.29,0.65)	0.45(0.23,0.90)
PPA + IDEXX	Apparent prevalence	0.47(0.44,0.50)	0.47(0.44,0.50)	0.47(0.44,0.50)	0.47(0.44,0.50)	0.47(0.44,0.50)
	True prevalence	0.33(0.30,0.36)	0.17(0.15,0.19)	0.11(0.09,0.13)	0.06(0.05,0.08)	0.02(0.01,0.03)
	Sensitivity	0.70(0.65,0.75)	0.92(0.87,0.95)	0.91(0.84,0.95)	0.91(0.81,0.96)	0.95(0.77,1.00)
	Specificity	0.65(0.61,0.68)	0.62(0.59,0.66)	0.59(0.55,0.62)	0.56(0.53,0.59)	0.54(0.51,0.57)
	Pos. predictive value	0.49(0.45,0.54)	0.33(0.29,0.37)	0.21(0.18,0.25)	0.12(0.09,0.15)	0.04(0.03,0.06)
	Neg. predictive value	0.82(0.78,0.85)	0.97(0.96,0.99)	0.98(0.97,0.99)	0.99(0.98,1.00)	1.00(0.99,1.00)
	Pos. likelihood ratio	2.00(1.78,2.26)	2.44(2.22,2.68)	2.19(1.99,2.41)	2.06(1.86,2.29)	2.09(1.87,2.34)
	Neg. likelihood ratio	0.46(0.38,0.54)	0.13(0.08,0.22)	0.16(0.09,0.28)	0.17(0.08,0.36)	0.08(0.01,0.57)
ParalisaTM + IDEXX	Apparent prevalence	0.50(0.47,0.53)	0.50(0.47,0.53)	0.50(0.47,0.53)	0.50(0.47,0.53)	0.50(0.47,0.53)
	True prevalence	0.33(0.30,0.36)	0.17(0.15,0.19)	0.11(0.09,0.13)	0.06(0.05,0.08)	0.02(0.01,0.03)
	Sensitivity	0.74(0.69,0.78)	0.92(0.87,0.96)	0.91(0.85,0.96)	0.91(0.81,0.96)	0.95(0.77,1.00)
	Specificity	0.62(0.58,0.65)	0.59(0.56,0.62)	0.55(0.52,0.59)	0.53(0.50,0.56)	0.51(0.48,0.54)
	Pos. predictive value	0.48(0.44,0.53)	0.31(0.27,0.35)	0.20(0.17,0.24)	0.11(0.08,0.14)	0.04(0.02,0.06)
	Neg. predictive value	0.83(0.79,0.86)	0.97(0.96,0.99)	0.98(0.97,0.99)	0.99(0.98,1.00)	1.00(0.99,1.00)
	Pos. likelihood ratio	1.93(1.73,2.16)	2.24(2.05,2.45)	2.05(1.87,2.24)	1.92(1.74,2.13)	1.96(1.75,2.19)
	Neg. likelihood ratio	0.43(0.35,0.51)	0.13(0.08,0.22)	0.15(0.09,0.28)	0.18(0.08,0.38)	0.09(0.01,0.60)

Table 1. Calculated test performance values and associated 95% confidence intervals (in parentheses) for two serological ELISA tests (IDEXX and Paralisa™), considered both singly and in combination, for a range of shedding threshold cutoffs determined using quantitative PCR in a dataset comprising 1,069 matched faecal and peripheral blood samples submitted for routine Johne's disease diagnosis.

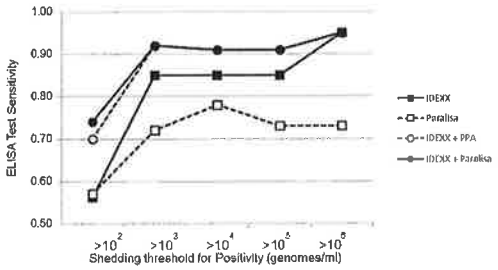

Figure 1. Test sensitivity data for two serological ELISA tests (IDEXX and Paralisa™), considered both singly and in combination, for a range of shedding threshold cutoffs determined using quantitative PCR in a dataset comprising 1,069 matched faecal and peripheral blood samples submitted to DRL for routine Johne's disease diagnosis.

Discussion

While recommendations for optimal diagnostic strategies can be made for NZ informed by international best practice and overseas studies, they must still be considered in light of cost and availability of diagnostic services available locally. In new Zealand, routine dairy herd screening for JD infection is optimally achieved by milk ELISA. In the absence of an organised control scheme judgements will continue to be made on a case by case basis and according to available resources. Due to the extreme demands placed on diagnostic tests by the dynamics of MAP infection and the persistent, chronic nature of Johne's disease progression in cattle, single tests used in isolation may be insufficiently exact to maximally inform management decisions particularly in the early, subclinical stages of disease when bioindicators are not present. Diagnostic tests based on ELISA, while quick and inexpensive are also amenable to the incorporation of additional antigens in order to broaden their diagnostic repertoire. In this study we observed a synergistic outcome through the incorporation of dual ELISA tests which resulted in an 18% and 17% increase in overall test sensitivity over individual tests used in isolation (IDEXX and Paralisa™, respectively) in a dataset where matched faecal samples reporting $>10^2$ genomes/ml (n=1,069) were considered positive and $\leq 10^2$ genomes/ml considered negative. Using a cutoff for positivity of $>10^3$ genomes/ml resulted in an increases of 7% and 20% (IDEXX and Paralisa™, respectively.

Although the nature of the antigen targeted by the IDEXX assay is proprietary, the observed increase in test sensitivity arose largely through the inclusion of protoplasmic antigen, a native protein derived from MAP and available as a proprietary reagent. While it is possible that the increased sensitivity observed through the inclusion of additional antigens arose at the cost of test specificity, true specificity values cannot be derived from this dataset as most samples were submitted from infected herds for routine testing. Diagnostic test protocols should be tailored to the prescribed outcomes of any management programme (Gardner *et al.* 2011), attempts to optimise diagnostic test sensitivity may not be necessary to achieve disease control (eradication), although increased test sensitivity may identify animals earlier, with subclinical infection.

In this study, quantitative faecal PCR was utilised as the gold standard methodology for the calculation of ELISA test performance data. While the number of reports of qPCR usage as a JD diagnostic assay is increasing, reflecting its increasing adoption

as a routine test methodology, often the quantitative data returned by this approach are not exploited to stratify shedding which is surprising. Of four MAP qPCR test kits marketed commercially (TetraCore VetAlert™, Life Technologies VetMAX™, AdiaVet ParaTB and Qiagen Bactotype® MAP PCR Kit) all interpret data qualitatively as a binary, positive or negative result when assay values fall below the prescribed maximal threshold cycle. The quantitative output of the DRL methodology described here also allowed test performance to be considered across a range to faecal shedding cutoffs.

While there is as yet no international agreement on standardisation of qPCR data for reporting of MAP faecal shedding (Marsh *et al.* 2014) in this study DNA standards were favoured for quantitation as they are quick and cheap to generate and, more importantly, they are constant, comparable and reproducible across laboratories, geography and over time. DNA standards are stable in storage and simple and cheap to prepare and quantify accurately, allowing a set of quantitation standards to be prepared that are highly reproducible from day to day and facilitate objective comparisons between samples over time. DNA standards serve as an immutable yardstick to provide relative quantitative judgements between samples, something which is difficult to achieve using mycobacterial cultures, artificial spiking of faecal samples or other standards which attempt to more closely mimic clinical samples. The benefits of a nucleic acid standard outweigh the fact that it is a surrogate standard; more important is that it provides a constant and universal measurement which does not change regardless of when or where prepared. The standards described here span the range of MAP shedding that we have observed in clinical samples and are linear in the assay over seven logs. While the use of a DNA standard to quantify MAP bacteria load in faecal samples is a necessary and pragmatic compromise between reporting an intuitive and biologically meaningful number (such as MAP bacilli or colony forming units/g of faeces) and having a robust, reproducible and constant comparator, their use does require a concession be made that the resulting units (genomes/ml) are a necessarily artificial construct.

Repeat findings show that multiple ELISA tests interpreted in parallel measurably increase sensitivity to diagnose MAP shedding and that the use of faecal qPCR as a serial test on ELISA positive animals allows for selective culling of MAP shedders without compromising overall test sensitivity. The use of laboratory diagnostics to manage JD in affected species is necessarily an exercise in compromise and investment in testing should reflect the goals of a management plan. While often lamented as lacking, extremes of JD test sensitivity may be counterproductive in some situations and particularly in cases of high prevalence where too many positive results may be overwhelming. In such instances, secondary testing may be used to stratify affected animals according to disease severity, facilitating prioritised culling decisions and limiting culling costs.

Abbreviations

MAP: *Mycobacterium avium* subsp. *paratuberculosis*; JD: Johne's disease; ELISA: Enzyme linked immunosorbent assay; EU: ELISA unit; PPD-J: Purified protein derivative – Johnin; PPA: Protoplasmic antigen; PCR: Polymerase chain reaction; qPCR: Quantitative polymerase chain reaction; PPV: Positive predictive value; NPV: Negative predictive value; HEYM: Herrold's egg yolk medium; NVSL: National Veterinary Services Laboratory.

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Diagnosis of sub-clinical infection with *Mycobacterium avium* subsp. *paratuberculosis* and its effect on milk production

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Objectives

A pilot project to determine if a novel diagnostic strategy coupling multi-antigen serological assay with faecal sampling for enumeration of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) shedding in faeces by quantitative PCR could be used within a commercial 1200 cow dairy herd in South Canterbury, New Zealand to identify and cull animals infected with MAP before clinical disease was evident. Secondary aims were to estimate the association between MAP status as defined by serological and faecal polymerase chain reaction (fPCR) and milk production.

Materials and methods

In the autumn of 2014 and 2015, a coccygeal tail vein blood sample was collected into a serum (red top) blood tube from all milking cows in the enrolled herd. At the Disease Research Laboratory (DRL), University of Otago, samples were assayed for circulating antibody to MAP by serum ELISA using a combination of two ELISA tests, Paralisa™ and IDEXX. The Paralisa™ test quantifies antibody responses to two distinct protein antigens in parallel, Johnin (PPD-J) and a Protoplasmic Antigen (PPA). An additional MAP-specific recombinant protein antigen, Ag_1Del_1, was incorporated into the Paralisa™ test and final results were arrived at by considering the three test antigens in parallel alongside the IDEXX ELISA assay. For all four serological antigens (Johnin, PPA, Ag_1Del_1, IDEXX) a classification of 'not detected' was returned for results of <50 ELISA units (EU), readings of 50–100 EU in any one test were classified as low, readings of 101–150 EU as moderate, and readings of >150 EU as high.

Seven days later, a single faecal sample (10g approx.) was collected from each cow testing low, moderate or high to any of the ELISA tests and forwarded to DRL for enumeration of MAP shedding in faeces by quantitative fPCR. Faecal sample data were stratified into shedding categories with MAP shedding scores of $\geq 10^3$–$<10^4$ genomes/ml classified as Moderate and counts exceeding $\geq 10^4$ genomes/ml as High. Faecal samples which returned shedding scores of $<10^3$ genomes/ml faeces were classified as Not Detected.

The outcome variable was the level of milk solids production (continuous, kg milk solids per cow) for the 2013–14 season and the 2014–15 season. Predictor variables were days in milk (continuous), age at sampling date in years (continuous), status as defined by the ELISA test (categorical, as above) and fPCR status (categorical, as defined above). A mixed model with a random intercept for cow and a fixed effect interaction term for year-ELISA gave the model with the lowest likelihood.

Results

The prevalence of ELISA positive cows was 297/1122 (26.5%, 95%CI=23.9–29.1%) in 2013–14 and 108/1069 (10.2%, 95%CI=8.4–12.0%) in 2014–15 (p<0.001). The prevalence of fPCR positive cows amongst these ELISA positive cows was 55/297 (18.5%, 95%CI=13.9–23.2%) in 2013–14 and 27/108 (25%, 95%CI=16.5–33.0) in 2014–15, (p=0.177).

Milk production was significantly greater in 2014–15 (473kgMS/cow 95%CI=466–479) than 2013–14 (430kgMS/cow (95%CI=424–435), p<0.001). Positive ELISA and faecal PCR were both significant in the final mixed model but there was a significant interaction between year and ELISA status such that the effect of ELISA status was greater in 2013–14 than 2014–15. Across both years the marginal mean production for cows with a high ELISA status was predicted to be 65kgMS/cow less and cows with a low or moderate ELISA status 39kgMS/cow less than cows with a non-detected ELISA status (p<0.001). Cows with a faecal PCR $\geq 10^3$ genomes/ml faeces produced 46kgMS/year less (p<0.001) than cows with a faecal PCR count $<10^3$.

Of the 297 ELISA positive cows in 2013–14, for 2014–15, 122 were culled, 61 were still positive and 114 were ELISA negative. Of the 55 cows testing positive for fPCR in 2013–14, for 2014–15, 52 were culled and three were still fPCR positive.

At the observed prevalence of fPCR in this herd, estimation of the sensitivity and specificity of the ELISA test for fPCR status suggest that the ELISA test is very useful for identifying animals that will have an fPCR $<10^3$ genomes/ml faeces with a negative predictive value = 92.6% (95%CI=87.2–97.9%).

Conclusions

In the absence of other clinical signs, herd screening using a combined ELISA test for MAP allowed identification of animals with a significantly decreased milk production. ELISA negative animals had a high probability of not being faecal shedders.

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